NUTRITION AND CANCER, *53*(2), 220–231
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Quercetin Induces Necrosis and Apoptosis in SCC-9 Oral Cancer Cells

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Abstract: *Evidence has accumulated that dietary polyphenols, in particular, flavonoids, have protective effects against oral cancer. In this study, we have examined the effects of quercetin, a major dietary flavonoid, on cell growth and necrosis/apoptosis and cell cycle regulation in human oral squamous carcinoma SCC-9 cells. Quercetin induced dose- and time-dependent, irreversible inhibition of cell growth and cellular DNA synthesis. Light microscopy and lactate dehydrogenase measurements showed modifications in the morphology and membrane integrity of these cells after quercetin treatment. Propidium iodide/annexin V staining showed that quercetin induced necrosis at 24 h and 48 h, whereas at 72 h cells underwent apoptosis, correlating with caspase-3 activation. Flow cytometry studies of the cell cycle distribution showed that quercetin induced mainly S-phase arrest. Thymidylate synthase (TS), a key S-phase enzyme, was inhibited in a time- and dose-dependent fashion by quercetin at the protein level. A lack of effect on TS mRNA suggested that TS down-regulation occurred at the translational level. In conclusion, our data support a view that quercetin initially induces a stress response, resulting in necrosis of these oral epithelial cells. Prolonged exposure of the surviving cells to quercetin causes apoptosis, presumably mediated by inhibition of TS protein.*

Introduction

Oral squamous cell carcinoma, the largest category of the head and neck cancers, represents approximately 3% of all cancers. Each year worldwide approximately 300,000 new cases are diagnosed with only a 50% survival rate over 5 yr. Common treatments, including surgery, radiation therapy and chemotherapy, have a very low success rate (1–3). The two chemotherapy drugs used most often are 5-fluorouracil (5-FU) and cisplatin (4). However, development of resistance and intolerable side effects to these drugs are major problems that need to be resolved. Epidemiological studies have clearly demonstrated a protective role of fruits and vegetables in oral cancer, presumably mediated by their content of polyphenols, particularly flavonoids (5–7). Thus, flavonoids might provide a supplementary approach in prevention/treatment of oral cancer.

Quercetin (3,5,7,3′,4′-pentahydroxyflavone) is the most abundant molecule in the extensive class of polyphenolic flavonoids ubiquitously found in plants and in many often-consumed foods, such as apples, onions, tea, berries, and many others (8–10). The average daily dietary intake of quercetin in a Western diet is estimated to be 16 mg/day (11), however, with a wide variability.

In cellular studies, quercetin has been found to have a broad spectrum of potentially beneficial biological activities, with a majority of studies focusing on protective effects against cancer. Thus, quercetin has been reported to inhibit the growth of cancer cells from the breast, lung, liver, ovaries, and colon. The molecular properties underlying these effects have been attributed to a wide array of biochemical mechanisms, including antioxidant properties (12) and effects on enzymes and signal transduction pathways involved in cell proliferation, cell cycle regulation, and apoptosis (13–16). Because of these potentially beneficial effects in combination with limited regulation of its human use, we now find quercetin being extensively employed in dietary supplements in doses as high as 1,000 mg/day.

A major obstacle for invoking health benefits of quercetin is its very low systemic bioavailability, mainly due to extensive metabolism by the intestine and liver (17), making it less likely to be useful against tumors in organs such as the breast, lung, liver, ovaries, and prostate. However, epithelial cells lining the digestive tract from the oral cavity to the intestine should have high exposure to quercetin and other flavonoids (18,19), be it through the diet or through food supplements.

For this reason, the focus of the present study was on the oral epithelium, using the oral squamous carcinoma SCC-9 cell line (20) as the model to examine the antiproliferative effects of quercetin, with special emphasis on properties of quercetin with direct functional significance for the oral cancer cell. Thus, our study examined the effect of quercetin on necrosis as well as apoptosis, cell cycle distribution, and on thymidylate synthase (TS), an important enzyme regulating

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DNA synthesis. Our observations provide evidence for early necrotic and sustained apoptotic effects of quercetin, which might be preventive as well as therapeutic in the fight against oral and potentially other cancers.

Materials and Methods

Materials

Quercetin dihydrate (>98% purity), bovine serum albumin (BSA), propidium iodide (PI), and 3-[4,5-dimethylthiazolyl-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal antibody against TS (TS-106) was obtained from Chemicon International (Temerula, CA). Goat anti-mouse secondary antibody was obtained from KPL (Gaithersburg, ME). [^{3}H]-Thymidine (20 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). All other chemicals were purchased from Sigma or Fisher (Pittsburgh, PA).

Cell Culture and Treatments

Human oral squamous carcinoma (tongue) SCC-9 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Fisher), supplemented with 10% fetal bovine serum (FBS) (Atlas Biologicals, Fort Collins, CO), 1% penicillin/streptomycin, and 0.4 µg/ml hydrocortisone in a 5% CO_2 atmosphere at 37°C. Vehicle dimethyl sulfoxide (DMSO, 0.1% final volume) was used as a control in all experiments. The cells were used at passage 10–30. The basal levels in untreated cells varied to some extent between different passages, but the magnitude of effects in the presence of quercetin was the same. All cell treatments except the thymidine incorporation assay were done in complete medium, that is, with 10% FBS.

Cell Proliferation Assays

The MTT assay measures cell viability and proliferation based on the ability of the mitochondrial succinate-tetrazolium reductase system to convert the yellow tetrazolium MTT to a purple formazan dye. The amount of dye produced is proportional to the number of metabolically live cells (21). Cells were seeded at an initial density of 5,000 cells/well in 96-well plates. After reaching 70–75% confluency, cells were exposed to quercetin (0–200 µM) in complete medium for 24 h, 48 h, or 72 h, each concentration being tested at least in triplicate. The medium was aspirated, and 100 µl MTT solution (0.5 mg/ml) was added for 3 h at 37°C to allow MTT metabolization (Sigma protocol). The formazan crystals were dissolved in 100 µl MTT solvent (0.1 M HCl, 10% Triton X-100 in anhydrous isopropanol). The absorbance of formazan formed was measured at 570 nm with 690-nm background subtraction using a microplate reader.

The thymidine incorporation assay measures the rate of proliferation of cells by determining the incorporation of [^{3}H]-thymidine into cellular nucleic acids (22,23). Briefly, cells were plated in 6-well plates at 0.2×10^6 cells/well, grown for 48 h, and then incubated with quercetin (0–50 µM) in serum-free medium with 0.1% BSA for 16 h. The cells were then pulsed with 1 µCi/µl of [3H]-thymidine for 3 h, washed, and trypsinized. After treatment with 5% trichloroacetic acid for 20 min on ice followed by washing with ethanol, the cells were lysed overnight. Acid-insoluble radioactivity was determined by liquid scintillation spectroscopy.

For recovery experiments, cells were incubated with 50 µM quercetin or vehicle for 48 h. The cells were then incubated for an additional 0–72 h in drug-free medium. After trypsinization, the number of cells was determined using a hematocytometer.

Hematoxylin–Eosin Staining

SCC-9 cells were treated in the presence or absence of 50 µM quercetin for 48 h. After fixation in 100% ethanol for 24 h, the cells were rehydrated with a graded series of decreasing concentrations of ethanol (95–0%), stained for 15 min with hematoxylin, washed with phosphate-buffered saline (PBS) for development of blue color, and then incubated with eosin for another 10 min. After staining, the cells were dehydrated with a graded series of increasing concentrations of ethanol (0–100%) and evaluated with a Zeiss Axiovert 100 microscope.

Lactate Dehydrogenase Leakage Assay

Quercetin toxicity in SCC-9 cells was determined at 24–96 h after the addition of 50 µM quercetin. The cells were harvested by scraping into 0.05 mM Tris/KCl buffer, pH 7.6, and were then sonicated. The cell lysate as well as the culture medium were analyzed immediately. Cell cytotoxicity in the cultures was assessed by determining release of lactate dehydrogenase (LDH) from the cells into the culture medium (24) using a kit from Pointe Scientific, Inc. (Lincoln Park, MI). LDH leakage was estimated from the ratio between the LDH activity in the culture medium and the total LDH activity (medium plus cell lysate).

Apoptosis Detection

Flow cytometry: The annexin V-FITC apoptosis detection kit (BD Pharmingen, San Jose, CA) was used for the detection of apoptotic cells (25) per manufacturer's specifications. Briefly, cells were incubated in the presence and the absence of 50 µM quercetin for 24, 48, and 72 h. After treatment, cells were washed twice with cold PBS, resuspended in 1X binding buffer at a concentration of 1×10^6 cells/ml, and then incubated with 10 µl of annexin V-FITC and 5 µl of

PI (1 μg/ml) for 15 min at room temperature in the dark. Samples were analyzed by flow cytometry within 1 h. Annexin V–positive/PI-negative cells were considered early apoptotic, annexin V– and PI-positive cells were late apoptotic, annexin V–negative/PI-positive cells were necrotic, and annexin V– and PI-negative cells were viable.

Fluorescence microscopy: The annexin V-FITC fluorescence microscopy kit coupled with PI staining was also used for the detection of adherent apoptotic cells. Briefly, cells were seeded on four-well chambered cover glasses and grown to about 75% confluency; this was followed by treatment with 50 μM quercetin or vehicle for 24, 48, or 72 h. Adherent cells were stained according to the kit instructions. The dual-labeled cells were visualized by fluorescence microscopy with a Leica TCS SP2 AOBS confocal microscope. Cells with bound annexin-V will show green staining in the plasma membrane. Cells that have lost membrane integrity will show red staining (PI) throughout the cytoplasm and a halo of green staining on the cell surface (plasma membrane). Cells with green staining were scored as apoptotic, cells with both green and red staining were scored as late apoptotic, whereas those with only red staining were considered necrotic.

Caspase-3 activity assay: A caspase-3/CPP32 Fluorometric Assay Kit (Biovision, Mountain View, CA) was used to determine the effect of quercetin on caspase-3 activation in SCC-9 cells according to the manufacturer's specifications. Briefly, cells at approximately 75–80% confluency were exposed to 50 μM quercetin or vehicle for 24–72 h. Suspensions containing 1×10^6 trypsinized cells/ml were incubated for 10 min on ice in lysis buffer followed by addition of 50 μM substrate (DEVD-7-amino-4-trifluoromethylcoumarin) (26) and incubation for 2 h at 37°C. The fluorescent proteolytic cleavage product was quantified by fluorometry with 400-nm excitation and 505-nm emission. Quercetin-induced changes in caspase-3 activity were normalized to the corresponding uninduced control activities.

Cell Cycle Analysis

Asynchronized cells were plated in 60-mm^2 plates at 0.1×10^6 cells/ml, grown until 75–80% confluency, and then treated with 0, 5, 10, 25, and 50 μM quercetin for 24, 48, and 72 h. The cells were collected by trypsinization, washed twice with cold PBS, and centrifuged. The pellets were resuspended in 100 μl cold PBS and 900 μl cold 70% ethanol and incubated overnight at 4°C. After centrifugation the cell pellets were washed with cold PBS and resuspended in PBS containing 1 mg/ml RNase and 100 μg/ml PI. Following incubation in the dark for 30 min at 4°C, the cells were analyzed by flow cytometry. Data were acquired on a BD FACSCalibur using CellQuest software and were analyzed using Modfit LT software.

TS Western Blotting

Cells grown to 75–80% confluency were treated with quercetin (5–50 μM) or DMSO in complete medium for 24–72 h. The cells were washed with cold PBS and lysed in ice-cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM NaF, 1 mM Na_3VO_4, 0.25% Na-deoxycholate, 1% NP-40, 1% Triton X-100, and 1 mM PMSF with freshly added protease inhibitors) for 10 min at 4°C, as modified from Ref. 27. The lysates were sonicated and cleared by centrifugation at 14,000 *g* for 15 min at 4°C, and the supernatant (total cell lysate) was used or immediately stored at –80°C. Protein concentration was determined by the Lowry assay (28). For Western blotting, 50 μg protein (boiled with sample buffer) was resolved on 12% Tris-HCl polyacrylamide gels (29) and transferred to nitrocellulose membranes (30) (Bio-Rad, Hercules, CA). The blot was blocked for 1 h with 5% milk in 0.1% Tris-buffered saline with 0.1% Tween-20 (TBST) and incubated with TS-106 monoclonal antibody (Chemicon International, Temecula, CA) (1:500) overnight at 4°C followed by washing with 0.1% TBST and incubation with HRP-conjugated goat antimouse secondary antibody (1:2,000) (KPL, Gaithersburg, MD). SuperSignal chemiluminescent substrate (Pierce, Rockford, IL) was added, and the membrane was exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ). The membrane was then stripped and probed for β-actin as a loading control. Densitometric quantitation of the bands used NIH Image 1.62 software.

Statistical Analysis

Data were expressed as mean ± SE. Statistical significance of differences between control and treated samples was calculated by Student's two-tailed *t*-test. $P < 0.05$ was considered significant. The IC_{50} values were calculated using Prism (GraphPad Software, San Diego, CA). Unless otherwise mentioned, all the data shown in this study are representative of two or three experiments.

Results

Effects on Cell Growth

To examine the antiproliferative effect of quercetin in SCC-9 cells, we first determined its effects on cell growth by the MTT assay, which measures the metabolically live cells based on their mitochondrial dehydrogenase activity (21). As shown in Fig. 1A, quercetin caused growth inhibition in a time- and dose-dependent manner. Quercetin produced statistically significant ($P < 0.05$) inhibition at 100 and 200 μM after 48 h treatment and at 50 to 200 μM after the 72 h treatment. The IC_{50} values were 94 μM and 50 μM for the 48-h and 72-h treatments, respectively.

In parallel, de novo DNA synthesis was measured as the incorporation of thymidine into DNA after 24-h treatment. As shown in Fig. 1B, the inhibitory effect of quercetin on

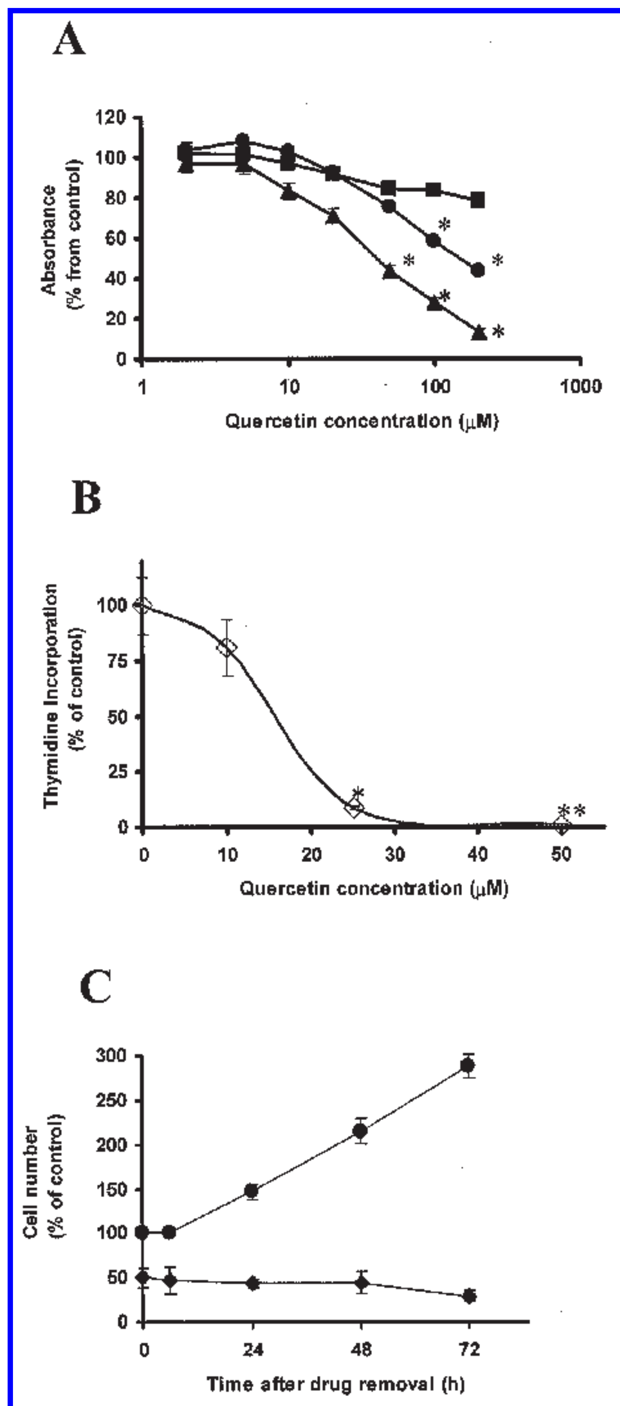

Figure 1. Antiproliferative effects of quercetin in SCC-9 cells. (A) The cells were incubated with quercetin (0–200 µM) for 24, 48, or 72 h in complete medium (with 10% fetal bovine serum). Cell proliferation was determined by the 3-[4,5-dimethylthiazolyl-2-yl]-2,5-diphenyltetrazolium bromide assay. Results are expressed relative to the 0-µM quercetin controls ■ 24 h, ● 48 h, ▲ 72 h). (B) The cells were incubated with quercetin (0–50 µM) for 24 h in serum-free medium with 0.1% bovine serum albumin. DNA synthesis was determined by the thymidine incorporation assay and expressed relative to the 0-µM quercetin control. (C) Cells were treated for 48 h with 50 µM quercetin or vehicle in complete medium. The medium was then replaced with drug-free medium, and after various times (0–72 h) the cells were trypsinized and counted. Results are expressed relative to control (48-h incubation with drug-free medium). ◆, quercetin; ■, control. All values are mean ± SE of at least triplicate cultures in two or three independent experiments. *, ** Different from control (P <0.05, 0.01).

DNA synthesis was much stronger than on cell growth, with a calculated IC_{50} value of 15 µM after only 24-h treatment.

To determine if the antiproliferative effect was reversible, cells were treated with 50 µM quercetin or vehicle for 48 h. The vehicle-treated and drug-treated cells were then incubated in drug-free medium for 0, 6, 24, 48, and 72 h followed by trypsinization and counting of the cells. After 48 h of treatment with quercetin, the cell number had decreased by 50% relative to vehicle-treated cells (time 0 in Fig. 1C). The different effect seen with direct counting of cells (Fig. 1C) compared with Fig. 1A may reflect that the MTT assay measures changes in mitochondrial activity, not directly the cell number. Once quercetin was removed, the cell number did not increase but rather showed a small further decrease, whereas the cell number in the absence of treatment, as expected, increased with time. These data indicate that the quercetin effect was not reversible.

Effects on Cell Morphology

Observations made under the light microscope showed that, after quercetin treatment, the cell number had decreased and, more interestingly, the shape of the cells had changed in comparison with control cells. To visualize more clearly these changes, cells were first incubated with 50 µM quercetin for 48 h and then fixed in ethanol, stained with hematoxylin and eosin, observed under the microscope, and photographed. As shown in Fig. 2A, in the cultures treated with quercetin, there were fewer cells, and, in the remaining cells, swelling and damage of the plasma membrane (indicated with arrows) could be seen.

To further assess the damage of quercetin to the plasma membrane, SCC-9 cells were treated with 50 µM quercetin for 24–96 h, and the leakage of intracellular LDH into the culture medium was measured (Fig. 2B). Treatment with quercetin for 24 h showed cell damage, causing a significant increase (1.8-fold) in LDH release into the culture medium, indicating that quercetin is cytotoxic. There was no further increase in the LDH release after longer treatment with quercetin. The effect on LDH release was concentration dependent with a significant increase also with 25 µM quercetin (data not shown).

Quercetin-Induced Necrosis Followed by Apoptosis

To determine if the quercetin inhibitory effects on cell growth and the morphological changes observed are due to necrosis and/or apoptosis, the cells were examined by annexin V-FITC and PI staining after treatment for 48 h with 50 µM quercetin. To visualize the necrotic/apoptotic features, the cells were analyzed by fluorescence microscopy. Figure 3 shows representative images of untreated and quercetin-treated cells. The untreated cells did not show any staining, suggesting that these cells do not undergo significant necrosis or apoptosis. Quercetin-treated cells displayed some staining for annexin V only (green; apoptotic cells) and

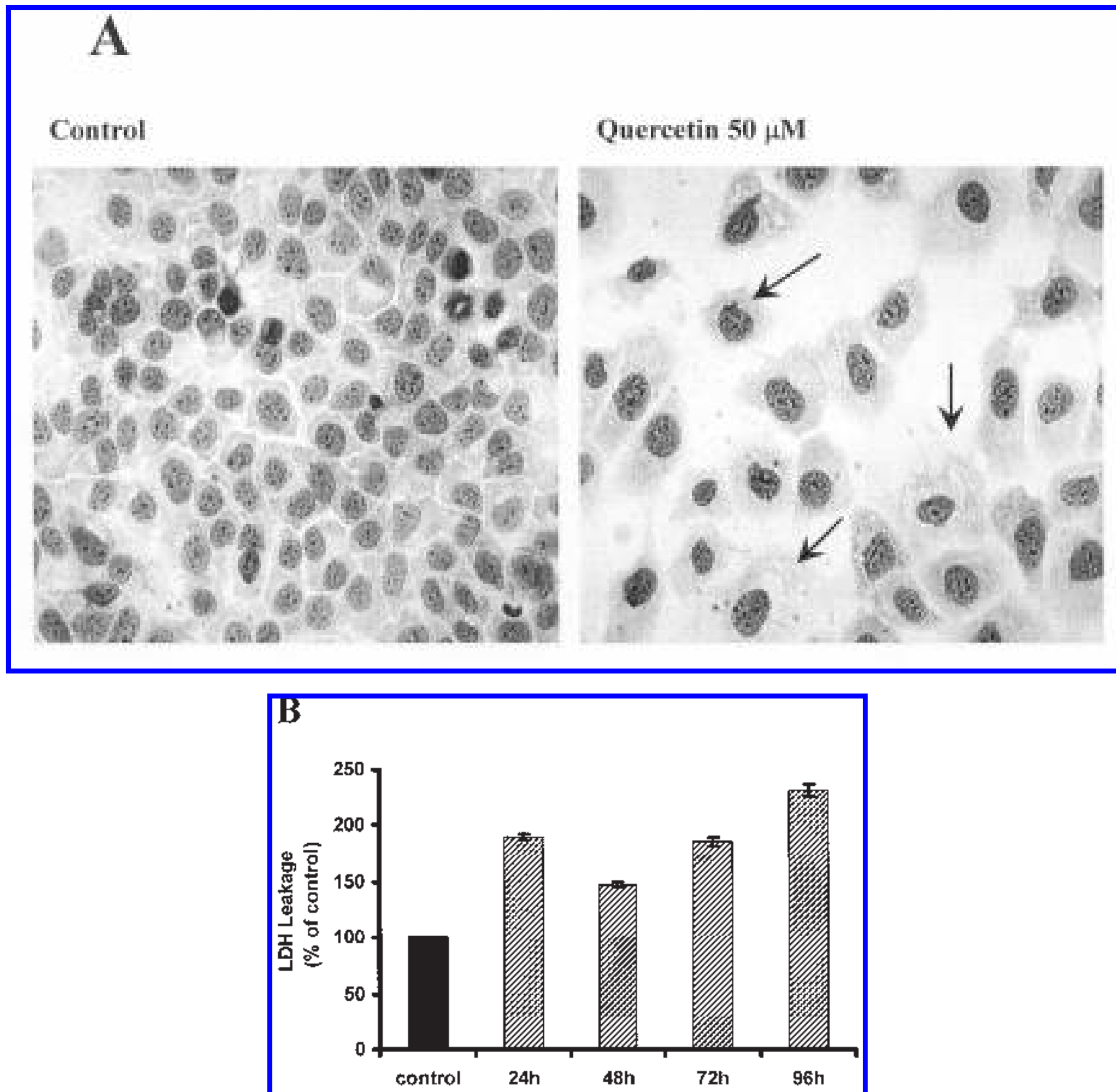

Figure 2. (A) Effect of quercetin on SCC-9 cell morphology. SCC-9 cells were exposed to 50 μM quercetin for 48 h in complete medium. After fixation, cells were stained with hematoxylin (blue nuclei) and eosin (pink cytosol) and then visualized under a light microscope at a magnification of 40×. (B) Effect of quercetin on the plasma membrane integrity. Cells were exposed to 50 μM quercetin for 24–96 h. Cell toxicity was determined by the release of lactate dehydrogenase (LDH) from the cells into the culture medium. LDH release is given as percentage increase relative to control (0.1% dimethyl sulfoxide). Values are mean ± SE of triplicates from three independent experiments. The quercetin-treated cells were different from control ($P < 0.05$).

more staining for both annexin V and PI (yellow; late apoptotic cells) and for PI only (red; necrotic cells).

To quantify the extent and time course of apoptosis and necrosis after quercetin treatment, we used flow cytometry after the same staining. The cells were sorted according to annexin V and PI status into early apoptotic, late apoptotic, necrotic, and viable cells. As shown in Fig. 4A, the number of early and late apoptotic cells after 24 and 48 h of quercetin treatment was not different from vehicle-treated controls (ratio ~ 1). In contrast, the number of necrotic cells increased 2.4- and 5.8-fold compared with controls at 24 and 48 h, respectively, and stayed elevated (4.2-fold) after 72 h. These data are consistent with the LDH leakage observed as early as after 24 h of treatment. After 72 h of treatment, the percentage of early and late apoptotic cells increased 6.2- and 3.2-fold, respectively, when compared with control, suggesting that at this late time point quercetin may induce an apoptotic effect that overlaps with the necrotic effect. It should be emphasized that the cell cycle analysis pertains to the cells that were still attached after the various treatments. It is likely that the apoptotic and particularly the necrotic effects are underestimated.

These data indicate that quercetin may induce an initial shock to the cells that results in necrosis followed by a "reorganization" of the remaining viable cells that submit themselves to apoptosis following prolonged treatment.

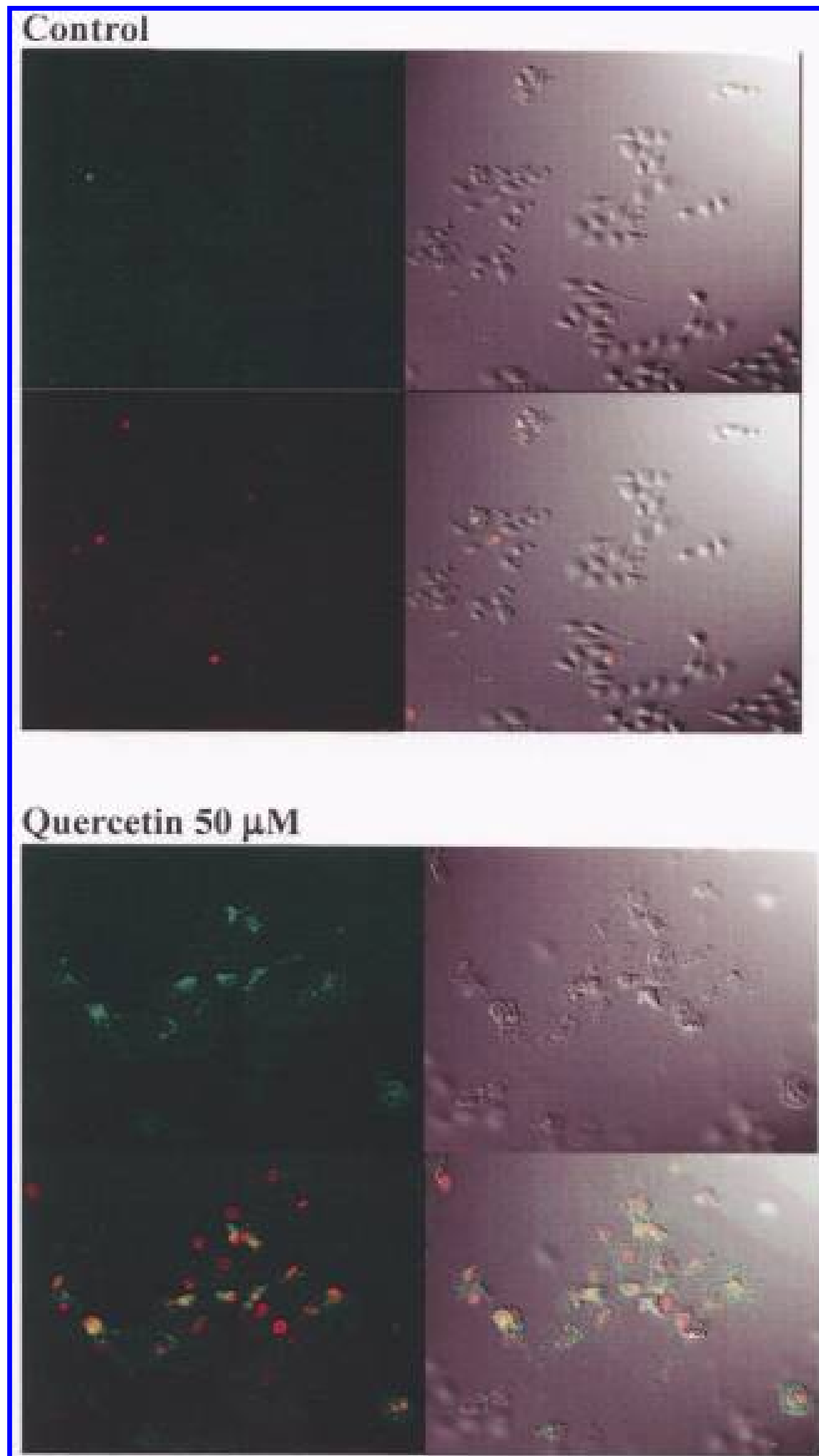

Figure 3. Effect of quercetin on SCC-9 cell necrosis and apoptosis. The cells were treated for 48 h with 50 μM quercetin or vehicle in complete medium. After staining, necrotic and apoptotic cells were detected by fluorescence microscopy (20×). Upper left panels, annexin V staining; upper right panels, phase contrast microscopy; lower left panels, annexin V and PI staining; lower right panels, overlapping phase contrast and annexin V and PI staining.

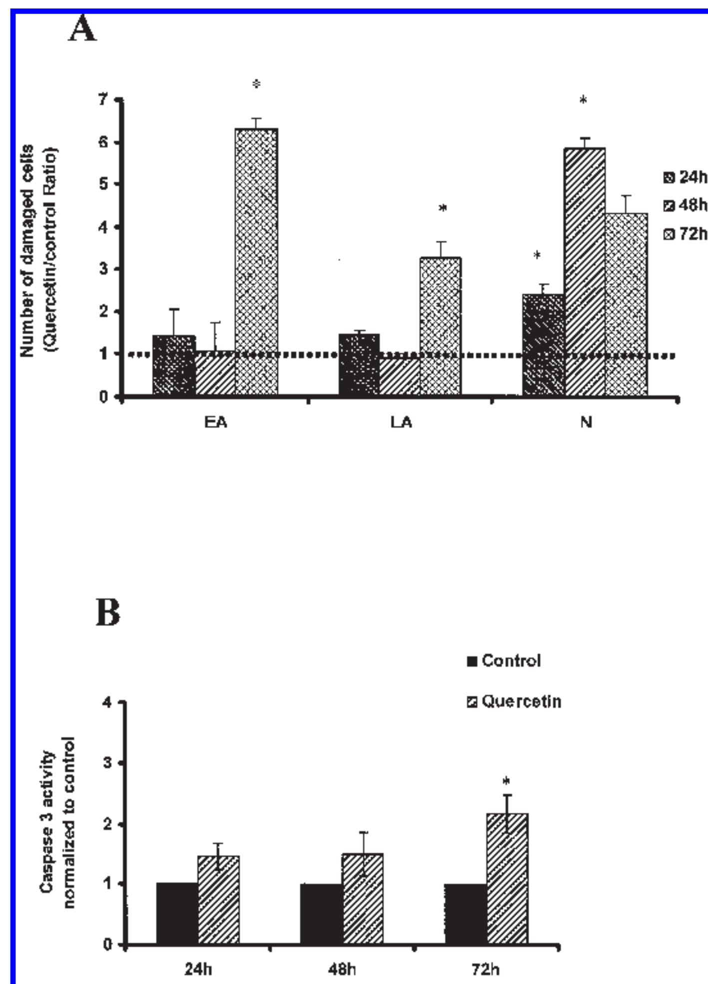

Figure 4. Effect of quercetin on necrosis and apoptosis. (A) SCC-9 cells were exposed to 50 µM quercetin or 0.1% dimethyl sulfoxide (DMSO; control) for 24, 48, and 72 h in complete medium, stained with annexin V and PI, and then analyzed by flow cytometry. The data are shown as the number of early apoptotic (EA), late apoptotic (LA), and necrotic (N) cells after quercetin treatment normalized to the corresponding control cells. Values are mean ± SE of duplicates from two independent experiments. *Different from control ($P < 0.05$). (B) Effect of quercetin on caspase-3 activation. SCC-9 cells were exposed to 50 µM quercetin for 24, 48, and 72 h in complete medium and then analyzed for caspase-3 activity. The activity after quercetin is shown as normalized to untreated controls (0.1% DMSO). Values are mean ± SE of duplicates from five independent experiments. *Different from the 72-h control ($P < 0.05$).

Caspase-3 Activation

SCC-9 cells were incubated in the absence and presence of 50 µM quercetin for 24, 48, and 72 h, and caspase-3 activity, a marker of apoptosis (31–33), was determined. As shown in Fig. 4B, after 24- and 48-h treatment quercetin did not significantly activate caspase-3, whereas after 72-h treatment quercetin induced a significant, 2.2-fold, increase in the activity relative to control. These data are consistent with the annexin V-PI flow cytometry data, which showed apoptosis after 72 h of treatment.

Induction of S-Phase Arrest

To assess whether quercetin-induced cell growth inhibition is mediated via alterations in cell cycle progression, asynchronized cells were incubated with increasing concentrations of quercetin (0–50 µM) for 48 h, and the effect of quercetin on cell cycle phase distribution was determined. A representative histogram is shown in Fig. 5A, and the data are summarized in Fig. 5B. When compared with control, consistent with its growth inhibitory effects, quercetin increased the population in the S phase with a corresponding decrease of cells in the G1 phase in a dose-dependent manner. In addition to increasing the population of cells in the S phase from 25.1% to 49.7%, 50 µM quercetin also appeared to increase the population in the G2-M phase, although this was not statistically significant. We did not detect any sub-G1 apoptotic peak, further confirming that the cells were not undergoing apoptosis after 48 h of treatment. These data corroborate the potent inhibitory effect of quercetin on DNA synthesis.

Inhibition of TS Expression

TS is a key enzyme in de novo DNA synthesis and is cell cycle regulated (34). As quercetin significantly inhibited DNA synthesis and induced S-phase arrest, we determined the effect of quercetin on TS expression. Cells were incubated with increasing concentrations of quercetin (0–50 µM)

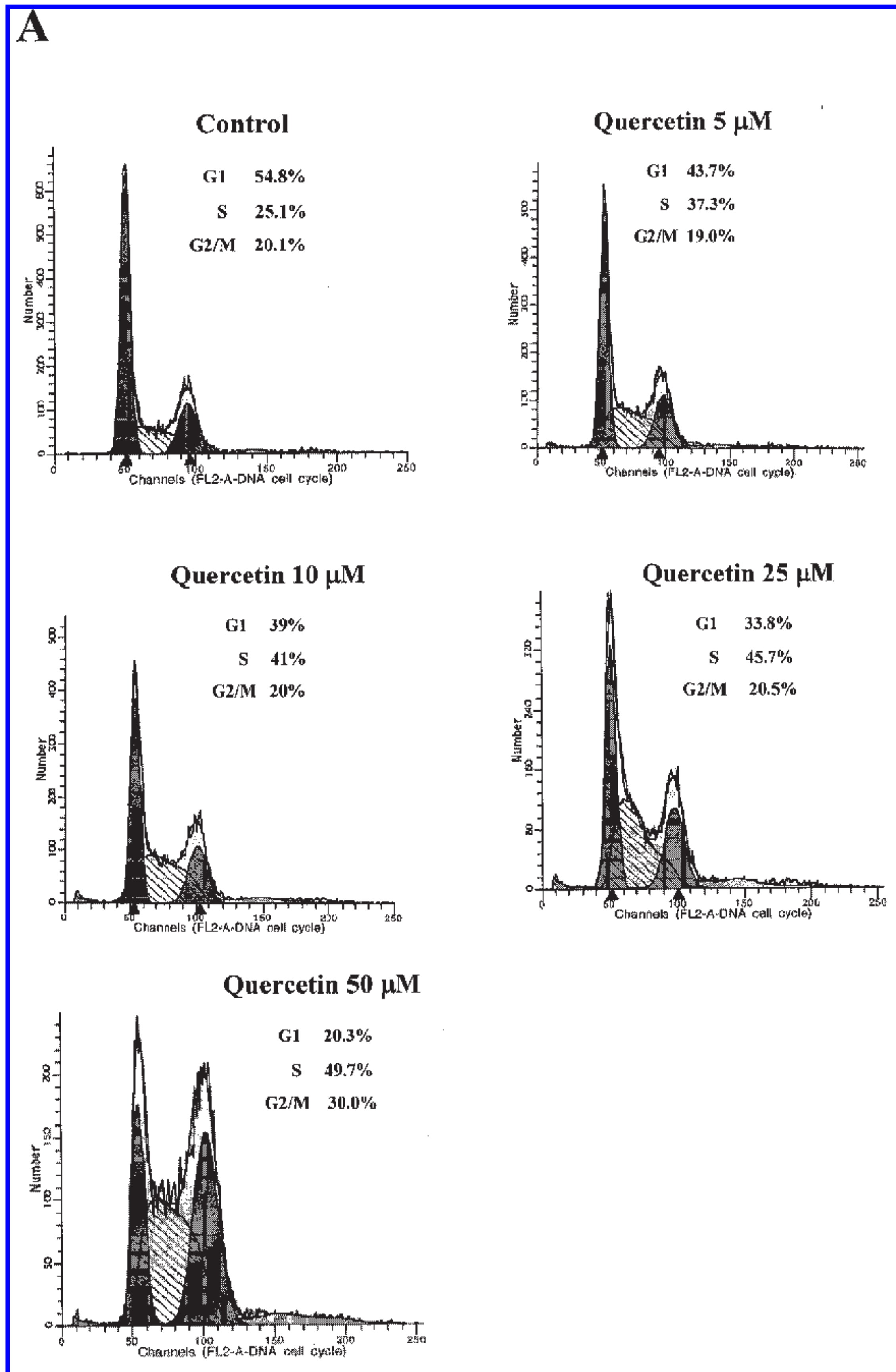

Figure 5. Effect of quercetin on cell cycle distribution. After treatment with 0, 5, 10, 25, or 50 μM of quercetin for 48 h in complete medium, cells were fixed and stained with propidium iodide, and the cell cycle distribution was analyzed by flow cytometry. (A) DNA histograms. (B) Plot of summarized values from DNA histograms. Two experiments were performed in duplicate and gave similar results.

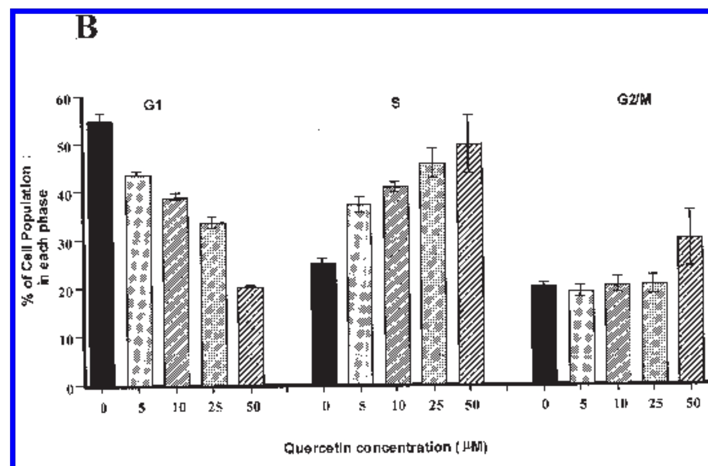

Figure 5. (Continued)

for 24, 48, and 72 h followed by immunoblotting of whole cell lysate using a TS monoclonal antibody. The positive control, MCF-7 cells treated with 50 μM 5-FU for 48 h showed both the TS ternary complex (upper band) and free TS (lower band) (35) (Fig. 6A). The ternary complex is an inactive form of TS, whereas the free form of TS is an active enzyme (27). Figure 6A shows that, whereas the expression of the free TS in untreated cells increased with time, quercetin inhibited free TS protein compared with the corresponding controls at all time points, with the 72-h treatment resulting in a 90% inhibition of free TS protein. Figure 6B shows that quercetin, after 48-h treatment, inhibited free TS expression in a dose-dependent manner, with 50 μM causing the greatest inhibition (88%). Interestingly, TS mRNA levels, as evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, were not significantly different after quercetin treatment compared with control (three separate experiments; data not shown).

Discussion

Squamous cell carcinoma is the most malignant neoplasm in the oral cavity and has the tendency to be aggressive if not discovered early. Traditional treatment consists of surgery followed by radiation and chemotherapy (1–3). 5-FU, alone or in combination with cisplatin, is the most commonly used chemotherapeutic drug (4). However, conventional chemotherapy is relatively ineffective and associated with numerous side effects, making it important to find new chemopreventive and/or chemotherapeutic tools that, added to the traditional treatments, may enhance the chance of survival for oral cancer patients.

Epidemiological studies suggest an inverse relationship between fruit and vegetable consumption and the risk of oral cancer (5–7). Such diets are rich not only in conventional nutrients but also in biologically active secondary metabolites, such as the flavonoids. These compounds display a large spectrum of biochemical properties related to chemoprevention of cancer (12–16). In this study we show that the major dietary flavonoid quercetin can inhibit oral cancer cell proliferation through induction of necrosis followed by apoptosis, through cell cycle changes, and through inhibition of TS expression.

Using as a model the oral SCC-9 cell line (20), we showed that quercetin inhibited oral cancer cell proliferation after 48 and 72 h of treatment (Fig. 1A) and significantly decreased DNA synthesis after 24 h of treatment (Fig. 1B). The much more potent effect of quercetin on DNA synthesis than on cell proliferation is noteworthy. The higher IC_{50} value seen in the MTT cell proliferation assay compared with the thymidine incorporation assay may be due to the higher protein concentration in the former assay, decreasing the free quercetin concentration (36). The difference in the time frame of effects may be explained by the fact that the MTT assay measures the effect on mitochondria, which is slower to develop than the effect measured by the thymidine incorporation. In a recent study, a biphasic response to quercetin was observed, with stimulation of cell proliferation for both colon and breast cancer cells at low concentrations of quercetin (37). This might have been due to an estrogenic effect of quercetin and was not seen with the oral cells in the present study. We also shown that, after treatment for 48 h, the effect of quercetin on cell number was irreversible (Fig. 1C). This may be attributed to the previous finding that quercetin undergoes cellular oxidation and covalent binding to macromolecules, including signaling proteins associated with cell proliferation (38).

Based on the effects on cell number and morphology (Fig. 2), our data demonstrated that quercetin was cytotoxic to the SCC-9 cells, and this effect was time and dose dependent, that is, treatment with low concentrations (5 and 10 μM) for short periods of times (24 h) was less cytotoxic (data not shown) than high concentrations (50 μM) for longer incubation times (48 h) (Fig. 2A). After quercetin treatment, cells became larger and their cell membranes showed clear breakdown in their integrity. The LDH assay confirmed that there was cell membrane damage when compared with untreated

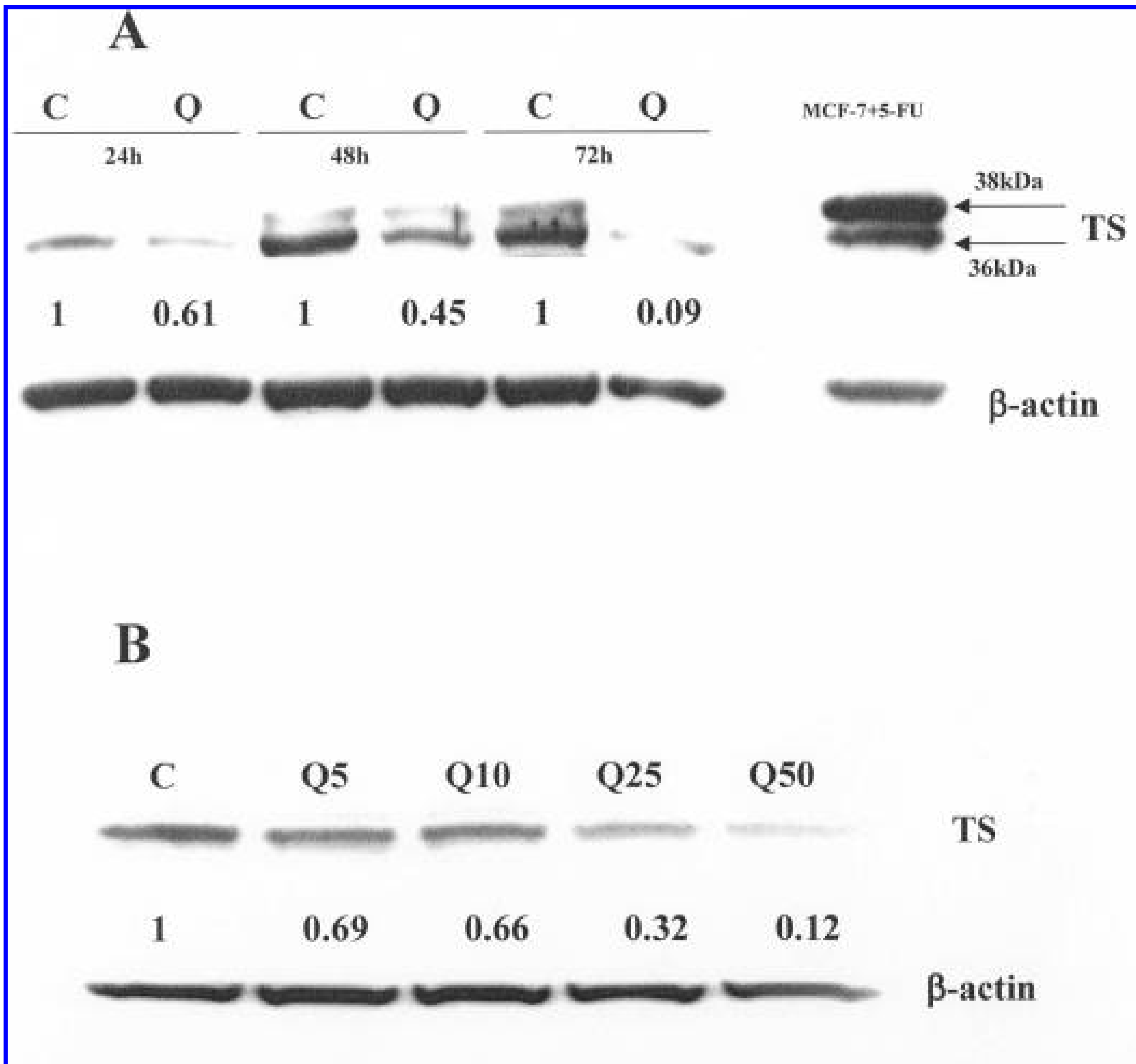

Figure 6. Effect of quercetin on thymidylate synthase (TS) expression. Western blot of TS protein in whole cell lysates following treatment with (A) 50 μM quercetin for 24, 48, and 72 h and (B) for 48 h with 0, 5, 10, 25, and 50 μM quercetin, both in complete medium. The upper band (~38 kDa) represents the TS ternary complex, whereas the lower band (~36 kDa) represents free TS. C, control; Q, quercetin; MCF-7+5-FU is the positive control, MCF-7 cells treated with 5-fluorouracil for 48 h. β-Actin was used as the loading control.

cells (Fig. 2B), suggesting a necrotic effect. In general, cells undergoing necrosis are swelling, with their membranes leaking, whereas apoptotic cells have their membranes intact, and the cells are shrinking. Although the distinction between apoptosis and necrosis can be easily observed, in practice the various death routes may overlap, and several characteristics may be displayed at the same time (39).

To distinguish between apoptosis and necrosis, cells were analyzed after staining with annexin V and PI. Fluorescence microscopy after this staining showed that, after 48 h of treatment, some quercetin-treated cells were positive for PI and some for both annexin V and PI (Fig. 3), suggesting that the cells at this time present necrotic and also apoptotic features. Interestingly, flow cytometric analysis of cells stained with annexin V and PI showed that, after 24 and 48 h of treatment, a significant increase in the number of necrotic cells could be seen, whereas, after longer incubation (72 h), a significant increase in early apoptotic cells could be observed (Fig. 4A).

This finding may be explained if treatment with quercetin is inducing an initial shock that kills some cells by necrosis. The remaining viable cells become resistant to quercetin treatment, and the necrosis process, a disorganized way of dying (39), is replaced by the cell-controlled death, the apoptosis process. The mechanism of the initial necrotic effect observed for quercetin is not understood. It might be due to a pro-oxidant effect, described in previous studies (40,41). However, based on the findings in this study, this effect is only transient, replaced by a more sustained apoptotic effect after longer treatment.

To confirm the apoptotic effect of quercetin after 72-h treatment, we determined if caspase-3, a key enzyme of the apoptotic process (31,32), is activated upon quercetin treatment. In accordance with the annexin V-PI staining, we observed that caspase-3 is significantly activated only after 72-h treatment (Fig. 4B), consistent with the possibility of initial necrotic shock followed by apoptosis.

Cell cycle analysis showed that quercetin blocked the cells in the S phase in a dose-dependent manner (Fig. 5), this effect being in accordance with the potent inhibition of DNA synthesis. S-phase arrest may occur at the expense of either G1 (42,43), as shown here in the SCC-9 cells, or G2/M (44), dependent of cell type and treatment conditions. Other studies have shown that quercetin can induce cell cycle arrest in the S phase (45) but also in the G1/S phase (43) or G2/M phase (46) depending on the cell type.

TS is an essential enzyme in de novo DNA synthesis, catalyzing the reductive methylation of deoxyuridine monophosphate to deoxythymidine monophosphate using 5,10-methylenetetrahydrofolate as the methyl donor (47). Because this is the only pathway that provides the essential thymidylate precursor for DNA synthesis, and because TS is highly expressed in oral cancer cells (48) and in patients diagnosed with oral cancer (49), TS represents an important and critical target in oral cancer chemotherapy. 5-FU, the most common drug used to treat oral cancer, arrests different types of cancer cells in the S phase of the cell cycle (42,44). 5-FU exerts its cytotoxic activity after its activation to 5-fluorodeoxyuridine 5′-phosphate FdUMP that forms a ternary complex with TS and 5,10-methylene tetrahydrofolate, resulting in TS inhibition and blockade of DNA synthesis (50).

In this study we showed that quercetin inhibited TS protein levels highly effectively, that is, about 90% at 50 µM, in a time- as well as dose-dependent manner (Fig. 6A, B). We did not see the formation of the ternary complex as with 5-FU (Fig. 6A, positive control), suggesting that quercetin inhibits free TS expression by an unknown mechanism, different from 5-FU. The time-dependent increase in TS expression in untreated cells is consistent with findings in other cell lines, where TS protein levels were maximal at two doubling times after plating (51). Considering that the doubling time in SCC-9 cells is around 40 h, these data corroborate the fact that TS expression indicates tumor cell proliferation. The inhibition of TS protein but not mRNA levels by quercetin treatment suggests that this is a translational effect.

Of practical significance is that quercetin has been shown to have access to the oral epithelial cells, either in the diet or through dietary supplements (18,19). Thus, its use as a chemopreventive agent either alone or in combination with drugs is a possibility. Such combinations could be with chemotherapeutic drugs currently in use in oral cancer treatment or possibly with more novel potential treatments such as with inhibitors of cyclooxygenase 2 (COX-2) and epidermal growth factor receptor (EGFR) (3,52). That quercetin-induced cell cycle arrest correlated with necrosis/apoptosis is important because in recent years cell cycle machinery and apoptosis as targets for intervention against cancer have been emphasized.

In conclusion, these data support a view that quercetin initially induces a stress response, resulting in necrosis of these oral epithelial cells. Prolonged exposure to quercetin of the remaining cells induces an apoptotic effect, presumably mediated by inhibition of TS. It should be pointed out that these effects may or may not be specific for the SCC-9 cells.

Acknowledgments and Notes

Supported by the Department of Defense/Hollings Cancer Center grant N6311602MD200 and the National Institutes of Health grant GM55561. We gratefully acknowledge the expert technical assistance by Louisa G. Carter, the valuable help with light microscopy by Dr. JoEllyn M. McMillan, and the important and constructive support from U. Kristina Walle in putting the manuscript together. We thank Dr. David Kurtz for valuable discussions. The Hollings Cancer Center Molecular Imaging Facility (Dr. Margaret Kelly) and MUSC Analytical Flow Cytometry Facility (Richard Peppler) were utilized in this study. This study was presented in part at the American Association for Cancer Research Special Conference: Cell Cycle and Cancer: Pathways and Therapies, Fort Lauderdale, FL, Dec. 1–5, 2004: Haghiac, M., J. T. Lucas, T. V. Bryant, U. K. Walle and T. Walle: Chemoprevention by polyphenols in oral cancer cells—antiproliferative effect and interaction with signaling pathways. Abstract # B30. Address correspondence to Thomas Walle, PhD, Department of Cell and Molecular Pharmacology and Experimental Therapeutics, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425. Phone: 843–792–2507. FAX: 843–792–2475. E-mail: wallet@musc.edu.

Submitted 21 July 2005; accepted in final form 18 October 2005.

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